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eIF4E Association with 4E-BP Decreases Rapidly Following Fertilization in Sea Urchin

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The eukaryotic translation initiation factor (eIF) 4F facilitates the recruitment of ribosomes to the mRNA 5′ end. The 4E-BPs are small proteins with hypophosphorylated forms that interact with the cap binding protein eIF4E, preventing its interaction with eIF4G, thereby preventing ribosome interaction with mRNA. In sea urchin, fertilization triggers a rapid rise in protein synthesis. Here, we demonstrate that a 4E-BP homologue exists and is associated with eIF4E in unfertilized eggs. We also show that 4E-BP/eIF4E association diminishes a few minutes following fertilization. This decrease is correlated with a decrease in the total amount of 4E-BP in combination with an increase in the phosphorylation of the protein. We propose that 4E-BP acts as a repressor of protein synthesis in unfertilized sea urchin eggs and that 4E-BP/eIF4E dissociation plays an important role in the rise in protein synthesis that occurs shortly following fertilization.

Key Words: **sea urchin fertilization; 4E-BP; eIF-4E; protein synthesis.**

INTRODUCTION

Sea urchin fertilization triggers a rapid and dramatic rise in protein synthesis independent of mRNA transcription and ribosome biogenesis (Brandhorst, 1976; Epel, 1967). Fertilization promotes the "unmasking" of stored mRNPs and consequent utilization of maternal mRNAs and ribosomes (Jenkins *et al.*, 1978; Kaumeyer *et al.*, 1978; Lopo *et al.*, 1989). In addition, in cell-free translation systems, the activity of several protein synthesis initiation factors is enhanced (Clemens, 1987; Colin *et al.*, 1987; Huang *et al.*, 1987; Lopo *et al.*, 1989), consistent with the increase of the rate of translation initiation in fertilized eggs. One critical factor is the eIF4F complex, whose activity is stimulated shortly following fertilization (Jagus *et al.*, 1992).

The eIF4F initiation factor facilitates the recruitment of ribosomes to the mRNA 5′ end (Raught *et al.*, 2000). It is composed of three subunits: 1) eIF4E, which recognizes and binds to the 5′ cap structure (m7GpppN, where N is any nucleotide); 2) eIF4A, an RNA-dependant ATPase and RNA helicase; 3) eIF4G, a large polypeptide that serves as a scaffold protein. eIF4F also interacts with the ribosome-associated initiation factor eIF3, and thus provides a physical link between the ribosome and the mRNA 5′ end, through the cap. The RNA helicase activity of eIF4A (in conjunction with eIF4B) is thought to facilitate ribosome binding by unwinding the mRNA secondary structure (Raught *et al.*, 2000).

eIF4E is a major target for regulation of translation initiation (Sonenberg and Gingras, 1998). eIF4E activity is thought to be regulated by phosphorylation of a conserved serine in vertebrates and invertebrates (Dyer *et al.*, 1998; Flynn and Proud, 1995; Joshi *et al.*, 1995; Whalen *et al.*, 1996). Phosphorylated eIF4E is reported to possess a higher affinity for the cap structure (Minich *et al.*, 1994) and to form a more stable eIF4F complex (Tuazon *et al.*, 1990). Furthermore, eIF4E activity is controlled by a family of translational repressors, the eIF4E binding proteins (or 4E-BPs, also called PHAS-I; *P*hosphorylated *H*eat- and *A*cid-*S*table protein, *I*nsulin stimulated). 4E-BPs compete with eIF4G for a common binding site on eIF4E (Mader *et al.*, 1995). Thus, 4E-BPs sequester eIF4E, and consequently prevent eIF4F complex assembly (Haghighat *et al.*, 1995).

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The affinity of 4E-BPs for eIF4E is determined by the phosphorylation status of the 4E-BPs. The underphosphorylated forms of 4E-BPs interact strongly with eIF4E and thereby inhibit cap-dependent translation, whereas the hyperphosphorylated forms do not (Pause *et al*., 1994). 4E-BPs become hyperphosphorylated in response to a large number of extracellular stimuli (Gingras *et al*., 1999a). In mammalian cells, phosphorylation of 4E-BPs is mediated by the PI3 kinase-related protein FRAP/mTOR (FKBP12 and rapamycin-associated protein/mammalian target of rapamycin) and the serine/threonine kinase Akt/PKB (protein kinase B) (Gingras *et al*., 1998; Gingras *et al*., 1999b). Three 4E-BP homologues exist in mammals: 4E-BP1, 4E-BP2, and 4E-BP3 (Pause *et al*., 1994; Poulin *et al*., 1998), and 4E-BP orthologs are found in numerous organisms including *Drosophila* and *Dictyostelium* (Gingras *et al*., 1999a), although only one 4E-BP has been described in *Drosophila* (Bernal and Kimbrell, 2000).

The isoelectric point of eIF4E shifts from 7.1 to 6.9 within 7 minutes following fertilization of sea urchin oocytes. This shift suggests a change in eIF4E phosphorylation (Waltz and Lopo, 1987), although such phosphorylation is likely not sufficient to increase eIF4F activity (Jagus *et al*., 1993). In addition, unfertilized eggs possess a repressor of eIF4F of yet unknown identity (Hansen *et al*., 1987; Huang *et al*., 1987), whose inhibitory activity is released gradually following fertilization (Jagus *et al*., 1992). Here, we report the existence of a 4E-BP homologue in sea urchin. Furthermore, we show that the amount of 4E-BP associated with eIF4E diminishes rapidly following fertilization. This decrease is a consequence of a decrease in the total amount of 4E-BP, in combination with its phosphorylation. We discuss the significance of these results in the cascade of reactions impinging upon the activation of translation that occurs following fertilization.

MATERIALS AND METHODS

Animals and Preparation of Gametes

Sphaerechinus granularis sea urchins were collected in the Brest area, kept in running sea water, and used within 5 days. Gametes collection and fertilization were performed as described previously (Meijer and Pondaven, 1988) with the following modification: eggs were dejellied by swirling 1 minute in filtered sea water (pH 5), and rinsed twice in filtered sea water prior to fertilization. Embryos were cultured at 16°C with constant stirring. At the indicated times following fertilization, cells were collected by centrifugation in a Sorvall TC6 centrifuge for 2 minutes at 2000 rpm and the cell pellet was frozen in liquid nitrogen and stored at −80°C.

Isolation of eIF4E from Unfertilized Eggs and Early Development Stages

Cell extracts were prepared and eIF4E was isolated as described previously (Xu *et al*., 1993). Briefly, cells were lysed in one cell volume of 2× binding buffer [40 mM Hepes (pH 7.4), 100 mM β-glycero-phosphate, 0.2 mM Na_3VO_4, 100 mM NaF, 10 mM ATP, 20 mM tetrasodium pyrophosphate (PPi), 100 mM NaCl, 0.4 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 2 mM DTT, 100 mM 4-(2-aminoethyl)-benzenesulfonylfluoride hydrochloride (AEBSF), and 20 μg/ml of aprotinin and leupeptin]. Lysates were centrifuged in a Sorvall RMC14 for 15 min at 14,000 rpm and at 2°C. Supernatants with an OD (260 nm) = 50 were mixed with 100 μl (25 μl bead volume) of m^7GTP-sepharose beads (m7GTP column) and incubated end-over-end for 60 min at 4°C. Unbound material was removed by six washes with 1 ml of binding buffer containing 100 mM NaCl. Bound proteins were eluted with 40 μl of binding buffer containing 100 mM NaCl and 0.4 mM m^7GTP for 10 min at 4°C. Following centrifugation at 2000 rpm for 2 min at 4°C, the supernatant was collected and stored in liquid nitrogen or mixed with Laemmli sample buffer. Typically, for SDS-PAGE and blots analyses, one-fifth of the purification was loaded.

Preparation of 4E-BP-Enriched Extracts

To enrich extracts in 4E-BP, cell lysates were boiled for 7 min at 100°C, incubated for 5 min on ice, centrifuged for 5 minutes at 14,000 rpm, and supernatants were recovered. Protein quantification was performed in duplicate by the Bradford assay. The proteins (500 μg) contained in the supernatant were precipitated with 10% TCA and recovered by centrifugation at 14,000 rpm for 10 minutes. The pellets were then washed twice with diethylether, dried, and resuspended in Laemmli sample buffer. Typically, one-tenth of the pellet was loaded on SDS-PAGE for subsequent analysis. Actin that copurifies in the heat soluble fraction was used as a loading control.

Immunological Analysis

The following antibodies were used: mouse monoclonal antibody directed against human eIF4E (Transduction Laboratories), rabbit polyclonal antibodies directed against human 4E-BP1 (Gingras *et al*., 1998), human 4E-BP2 (Rousseau *et al*., 1996), and *Drosophila* 4E-BP (Miron *et al*., 2001). Anti-mouse actin antibodies were purchased from Upstate Biotechnology. Secondary antibodies coupled to peroxidase were obtained from Dako S.A. (France).

For immunoprecipitation analysis, cell lysates or proteins purified using a m7GTP column were incubated with anti-4E-BP2 antiserum (1:250) at least 4 h at 4°C, in 1 ml Tris-buffered saline buffer (pH 7.5) containing 100 mM β-glycero-phosphate, 1 mM AEBSF, and 20 μg/ml of aprotinin and leupeptin. The antigen–antibody complex was incubated with protein A-Sepharose beads for 1 h at 4°C and the resin was washed three times with 50 mM Tris–HCl (pH 7.5), 150 mM KCl, 1% NP-40, 1 mM EDTA and twice with 50 mM Tris–HCl (pH 7.5), 150 mM KCl.

Western blot analyses were performed following electrotransfer of proteins onto 0.22-μm nitrocellulose membranes (Towbin *et al*., 1979). Membranes were incubated with anti 4E-BPs, anti eIF4E (1:2000), or anti-actin (1:10,000) antibodies in 5% skim milk, 0.1% Tween 20, 20 mM Tris–HCl (pH 7.6) at room temperature. The antigen–antibody complex was revealed by the chemiluminescence system (ECL; Amersham) with peroxidase-coupled secondary antibodies according to the manufacturer's instructions. Signals were quantified using the public domain NIH Image program (written by Wayne Rasband at the U.S. National Institutes of Health). When equal loading was necessary, amounts of eIF4E or 4E-BP were first compared by Western blot analysis and adjusted accordingly for following blots.

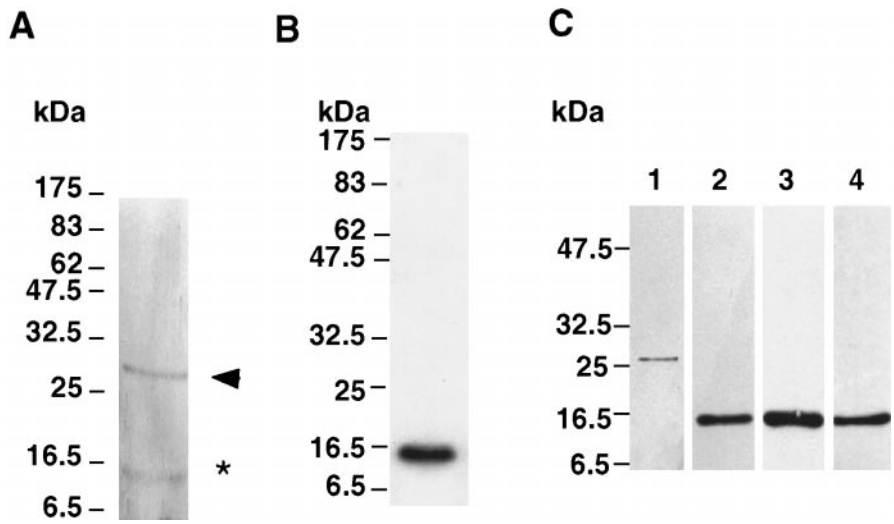

FIG. 1. Identification of eIF4E and 4E-BP in unfertilized sea urchin eggs. (A) Unfertilized eggs extract was applied to a m^7GTP-column and bound proteins were eluted from the column using the cap analogue m7GTP and visualized by Ponceau red staining following 12% SDS-PAGE and electrotransfer onto a nitrocellulose membrane. (B) Far-Western analysis using ^{32}P-labeled mouse recombinant HMK-eIF4E as a probe. (C) Western blot analysis using antibodies directed against human eIF4E (lane 1), human 4E-BP1 (lane 2), human 4E-BP2 (lane 3), and *Drosophila* 4E-BP (lane 4).

Acid Phosphatase Treatment

Sea urchin unfertilized and fertilized eggs extracts were immunoprecipitated as described above, and beads were washed three times with phosphatase buffer [50 mM Mes (pH 6.0), 0.1% β-mercaptoethanol, 100 mM AEBSF, and 20 μg/ml of aprotinin and leupeptin]. The beads were then exposed to 2 units/ml of potato acid phosphatase (Boerhinger grade II) for 15 minutes at 30°C. At the end of the incubation, the beads were washed twice with 50 mM Tris–HCl (pH 7.5), 150 mM KCl.

Far-Western Analysis

Far-Western blotting were performed using ^{32}P-labeled, HMK-tagged recombinant murine eIF4E, as described previously (Pause *et al.*, 1994). Briefly, proteins were separated on an SDS-PAGE and transferred onto a nitrocellulose membrane. Membranes were preincubated in HBB buffer [25 mM Hepes-KOH (pH 7.7), 25 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, 0.1% NP-40, and 5% skim milk] 2 h at 4°C and then incubated in Hyb75 buffer [20 mM Hepes-KOH (pH 7.7), 75 mM KCl, 2.5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 0.1% NP-40, and 1% skim milk] containing ^{32}P-labeled HMK-eIF4E (250,000 cpm/ml) overnight at 4°C. After extensive washing in Hyb75 buffer, the membrane was dried, autoradiographed, and quantified as described above.

2D Gel Analysis

Immunoprecipitated 4E-BP (see above) was resuspended in 2D sample buffer [9 M urea, 2% β-mercaptoethanol, 0.8% Pharmalytes (pH 3–10), 0.5% Triton X-100] and separated onto Immobiline DryStrip gel (pH 3–10), as described by the manufacturer (Amersham Pharmacia Biotech). For the second dimension, DryStrip was equilibrated in 100 mM Tris (pH 6.8), 6 M urea, 30% glycerol, 1% SDS, for 10 minutes in buffer containing 65 mM DTT, and 10 minutes in buffer containing 0.25 M iodoacetamide, loaded onto 12% SDS-PAGE, transferred onto a nitrocellulose membrane, and immunodetected using anti-4E-BP2 antibody and ECL reagents (see above).

RESULTS

Identification of 4E-BP in Unfertilized Sea Urchin Eggs

To search for a possible translational repressor bound to eIF4E in unfertilized eggs, we purified sea urchin eIF4E by affinity chromatography and identified copurified proteins. Extracts prepared from unfertilized eggs were passed through a m^7GTP affinity gel, and retained proteins were eluted with an excess of the cap analogue m^7GTP. Following resolution of the eluted proteins by SDS-PAGE and transfer to nitrocellulose membrane, Ponceau red staining of the membrane revealed the presence of two major polypeptides with apparent molecular weights of 28 kDa (Fig. 1A, arrow) and 16 kDa (asterisk). Next, purified proteins were tested for eIF4E binding by far-Western blotting using recombinant ^{32}P-labeled HMK-eIF4E as a probe (Pause *et al.*, 1994). Among the proteins retained on the m7GTP column, a single 16-kDa polypeptide interacted strongly with radiolabeled eIF4E (Fig. 1B). Based on these new data, we suspected that we had purified eIF4E and 4E-BP homologues of sea urchin. To test the identities directly, specific antibodies directed against eIF4E and 4E-BPs were used. As expected, the 28-kDa protein was specifically recognized by a monoclonal antibody directed against rabbit-eIF4E (Fig.

1C, lane 1) and therefore identified as *S. granularis* eIF4E. Also, three distinct antibodies directed against human 4E-BP1, human 4E-BP2 (Rousseau *et al*., 1996), and *Drosophila* 4E-BP (Miron *et al*., Submitted for publication), recognized a single polypeptide with an apparent molecular weight of 16 kDa (Fig. 1C, lanes 2–4). These data indicate that both eIF4E and 4E-BP orthologues exist in sea urchin and that 4E-BP is associated with eIF4E in unfertilized eggs, as both proteins are retained on a m7GTP column.

Coimmunoprecipitation of 4E-BP Bound to eIF4E

While at least three 4E-BP homologues possessing distinct molecular weights are found in mammalian cells (Pause *et al*., 1994; Poulin *et al*., 1998), our experiments revealed that three different antibodies recognized a single 16-kDa polypeptide in sea urchin. We then asked which of these antibodies was the most efficient to detect or to immunoprecipitate sea urchin 4E-BP. To address this question, proteins were first purified from unfertilized eggs using a m7GTP column, and then subjected to immunoprecipitation followed by immunodetection with various antibodies. Immunoblotting with antibodies specific to human 4E-BP1 following immunoprecipitation using either anti-human 4E-BP1 or anti-4E-BP2 antibodies revealed in both cases a single protein with an identical mass of 16 kDa (Fig. 2A, lanes 1–5). Similar results were obtained in the cross-immunodetection experiment using anti-human 4E-BP2 and anti-drosophila 4E-BP antibodies (data not shown). Among the antibodies, the anti-human 4E-BP2 antibody was the most efficient for both immunodetection (Fig. 1C, compare lane 3 to lanes 2 and 4) and immunoprecipitation (Fig. 2A, compare lane 5 to lane 3) of sea urchin 4E-BP. Furthermore, it is able to coimmunoprecipitate eIF4E bound to 4E-BP, which has been previously purified from unfertilized eggs using a m7GTP column (Fig. 2B, compare lane 3 to lanes 1 and 2). We then decided to use the anti-4E-BP2 to detect 4E-BP in the following experiments.

All the experiments described above have been performed using proteins which were first passed through a m7GTP column. To ensure that we could immunoprecipitate and detect 4E-BP directly, total extracts prepared from unfertilized eggs were incubated in the presence of the anti-human 4E-BP2 antibody coupled to Sepharose beads, and immunoprecipitated proteins were revealed by Western and far-Western blotting. 4E-BP was efficiently immunoprecipitated from total extracts (Fig. 3A, compare lane 3 to lanes 2 and 1) and immunoprecipitated 4E-BP bound to radiolabeled eIF4E (compare lane 6 to lanes 4 and 5). Similarly, direct Western and far-Western blotting revealed that the anti-human 4E-BP2 antibody recognized sea urchin 4E-BP (Fig. 3B, lane 1) and radiolabeled eIF4E bound to 4E-BP (lane 2) in extracts enriched in 4E-BP. The facts that 1) sea urchin 4E-BP is recognized by antibodies directed against its human counterpart, 2) sea urchin and mammalian 4E-BPs possess similar biochemical properties (heat and acid stability), and 3) sea urchin 4E-BP interacts directly with

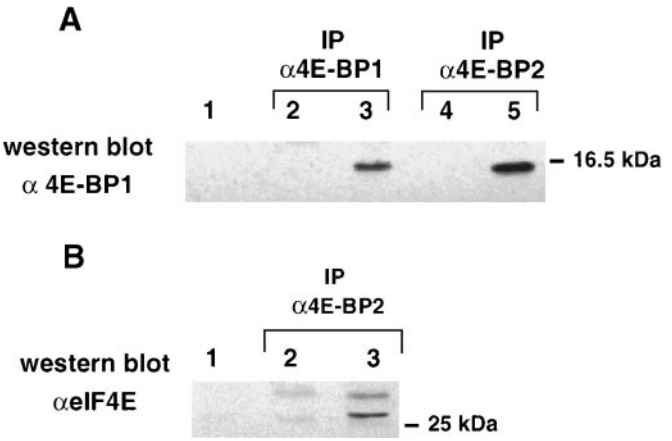

FIG. 2. Coimmunoprecipitation of eIF4E with 4E-BP. (A) Extracts prepared from unfertilized eggs were first passed through a m^7GTP column. Following elution with m^7GTP, recovered proteins were immunoprecipitated with anti-human 4E-BP1 (lane 3) or anti human 4E-BP2 (lane 5) antibodies. Immunoprecipitates were resolved on 15% SDS-PAGE and a Western blot was performed using anti human 4E-BP1 antibody. Protein A sepharose beads alone (lane 1) and anti-human 4E-BP1 (lane 2) or anti-human 4E-BP2 (lane 4) antibodies alone were used as controls. (B) Proteins obtained as described in A were immunoprecipitated with anti human 4E-BP2 antibody (lane 3), resolved by 15% SDS-PAGE and a Western blot was performed using anti-human eIF4E antibody. Protein A sepharose beads alone (lane 1) and anti human 4E-BP2 antibody alone (lane 2) were used as controls.

mouse recombinant eIF4E by far-Western, strongly suggest that 4E-BP protein is highly conserved from sea urchin to mammals. Since 4E-BP/eIF4E interaction can be detected directly from egg extracts, we decided to monitor 4E-BP/eIF4E interaction following fertilization.

4E-BP/eIF4E Interaction Decreases Following Fertilization

In mammalian cells, the stability of the eIF4E/4E-BP complex is under the control of a large array of extracellular stimuli that impinge upon cap-dependent translation initiation. As translation is strongly activated following fertilization of sea urchin eggs, it is conceivable that the association between eIF4E and 4E-BP is disrupted following fertilization. To test this hypothesis, eIF4E/4E-BP interaction was first analyzed in total extracts by far-Western blotting at different times following fertilization. Indeed, recombinant eIF4E bound strongly to 4E-BP in extracts prepared from unfertilized eggs, while the interaction decreased dramatically within 15 minutes following fertilization (Fig. 4A, compare lane 1 to lane 2) and remained very low up to 45 minutes (Fig. 4A, lanes 2–4). Similar results were obtained with eggs isolated from three different females (Fig. 4B). These data indicate that the interaction between sea urchin 4E-BP with recombinant eIF4E de-

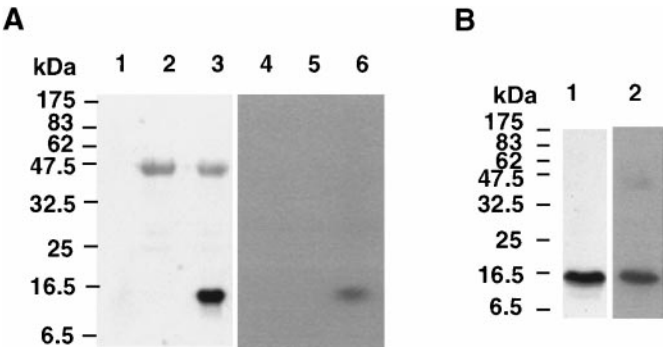

FIG. 3. Western and far-Western analysis of 4E-BP in total extracts. (A) Total extracts from unfertilized sea urchin eggs were incubated in the presence of anti-human 4E-BP2 antibody coupled to protein A-sepharose beads. Immunoprecipitated proteins were resolved by 15% SDS-PAGE and revealed by Western blot using the same antibody (lanes 1–3) or by far-Western analysis (see Materials and Method) using ^{32}P-labeled mouse recombinant HMK-eIF4E as a probe (lanes 4–6). Protein A-sepharose beads alone (lanes 1 and 4) and anti human 4E-BP2 antibody alone (lanes 2 and 5) were used as controls. (B) Extracts were enriched in 4E-BP (see Materials and Methods) and 4E-BP was revealed by Western (lane 1) and far-Western (lane 2).

creases following fertilization. We then asked whether 4E-BP/eIF4E interaction is also modified *in vivo*. To adress this question, 4E-BP bound to eIF4E was analyzed after affinity purification of eIF4E on m7GTP columns (Fig. 4C). The amount of 4E-BP associated with eIF4E that bound to a m7GTP column diminished following fertilization (Fig. 4C, compare lane 1 to 2). According to our hypothesis, these data suggest that sea urchin fertilization promotes a decrease in 4E-BP/eIF4E interaction.

The decrease in eIF4E/4E-BP interaction likely reflects changes of 4E-BP protein rather than in eIF4E. To test this possibility, equal amounts of 4E-BP immunoprecipitated from total extract prior to and following fertilization (Fig. 4D, top panels) were examined for eIF4E binding (Fig. 4D, bottom panels). A far-Western analysis showed that eIF4E interacted less efficiently after fertilization (Fig. 4D, compare lane 1 to lane 2 and 3). These data suggest a loss in 4E-BP affinity for eIF4E following fertilization.

We also analyzed the interaction between eIF4E and 4E-BP immediately after fertilization. Extracts enriched in 4E-BP from eggs prior to and every 2 minutes following fertilization were analyzed by far-Western. eIF4E/4E-BP binding diminishes a few minutes following fertilization (Fig. 5A, top panel). Strikingly, although equal amount of total proteins was loaded, as judged by anti-actin Western blotting (Fig. 5A, bottom panel), the amount of 4E-BP diminished after fertilization (middle panel). However, analysis of equal amounts of 4E-BP immunoprecipitated prior to or 15 minutes after fertilization revealed that the decrease of 4E-BP amount was not sufficient to explain the dramatic loss in eIF4E/4E-BP interaction, as revealed by immunoprecipitation (Fig. 5B). The loss of eIF4E/4E-BP binding suggests that 4E-BP is subjected to a modification following fertilization, most likely phosphorylation (Pause *et al.*, 1994).

Fertilization Promotes Phosphorylation of 4E-BP

To test whether 4E-BP is phosphorylated following fertilization, 4E-BP immunoprecipitated from egg extracts was separated by two-dimensional gel electrophoresis and subjected to anti 4E-BP2 Western blotting. In unfertilized eggs, two major and two minor spots were detected by the anti-4E-BP2 antibody (Fig. 6, top panel left, spots numbered from 1–4, with a p*I* of 6.0, 5.4, 5.3, and 4.9, respectively). Most of the signal was concentrated in less acidic spots (1 and 2) prior to fertilization (top panel, left). However, the signal shifted dramatically to more acidic forms (spots a and b, p*I* 5.4 and 5.1, respectively) after fertilization (bottom panel, left). To demonstrate that the shift from high to low p*I* spots was due to phosphorylation, 4E-BP immunoprecipitated before and after fertilization was treated with acid phosphatase (Fig. 6, right panels). In unfertilized eggs, the two major spots (1and 2) were still detected following acid phosphatase treatment. The two minor spots (3 and 4) disappeared and two new spots (c and d) appeared at higher p*I*s and lower molecular weights, suggesting that 4E-BP is partly phosphorylated before fertilization. Furthermore, acid phosphatase treatment of 4E-BP from fertilized eggs generated a similar pattern, demonstrating that 4E-BP is phosphorylated early after fertilization. Thus, the dramatic decrease in eIF4E/4E-BP interaction that occurs shortly following sea urchin eggs fertilization is correlated with 4E-BP hyperphosphorylation.

DISCUSSION

The dramatic rise in the rate of translation initiation that occurs shortly following fertilization of sea urchin eggs has been partly explained by an augmentation in the activity of the cap-binding complex, eIF4F (Lopo *et al.*, 1989; Jagus *et al.*, 1992). Interestingly, it was suggested that eIF4F activity was repressed in unfertilized eggs owing to the presence of an inhibitor of unknown identity that could block eIF4F assembly through a direct interaction with eIF4E (Jagus *et al.*, 1993). Here, we provide evidence that this inhibitor is the sea urchin homologue of 4E-BP, the repressor of eIF4E function well characterized in mammalian cells.

Diverse antibodies directed against mammalian and *Drosophila* 4E-BPs recognized 4E-BP from sea urchin. Furthermore, mammalian eIF4E binds sea urchin 4E-BP by far-Western analysis. These data suggest that 4E-BPs are highly conserved through evolution, from sea urchin to mammals. The fact that all the antibodies we have tested gave a single signal with an apparent molecular mass of 16 kDa might suggest the presence of a single ancestral 4E-BP gene in sea urchin. However, these experiments do not rule out the

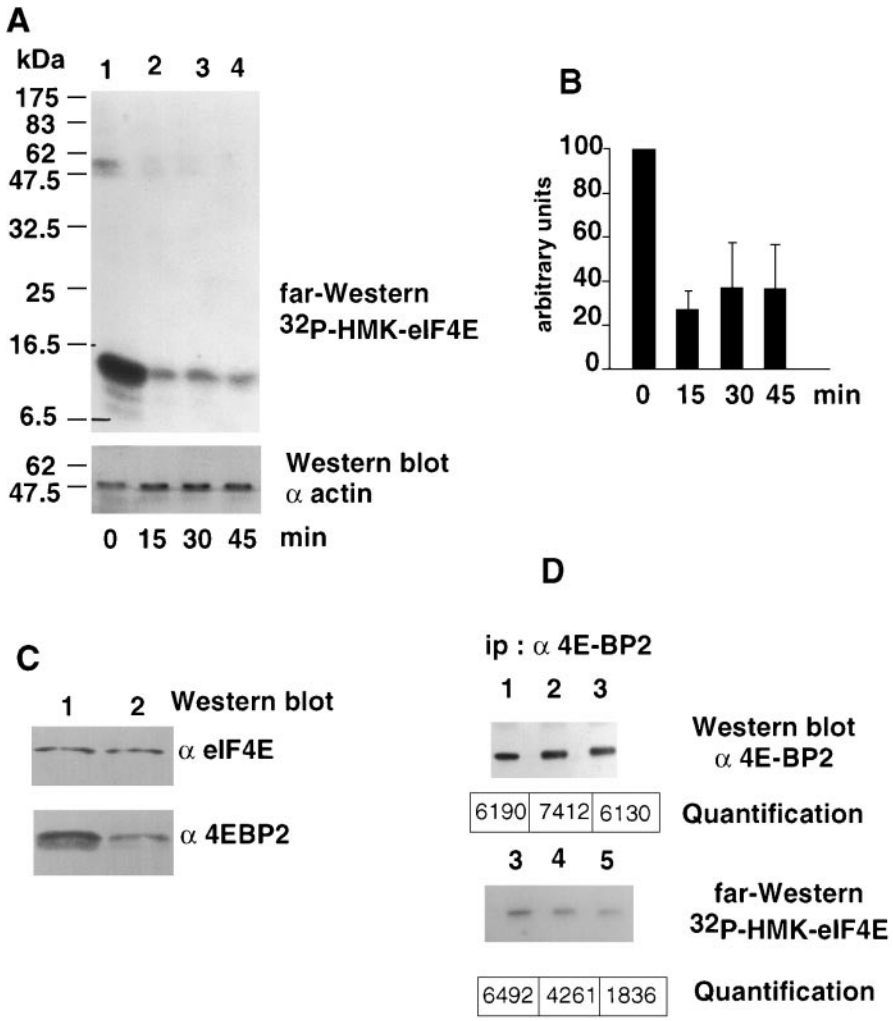

FIG. 4. eIF4E/4E-BP association decreases following fertilization. (A) Far-Western analysis. Enriched extracts (see Materials and Method) prepared prior to or every 15 minutes following fertilization (as indicated) were resolved on 15% SDS-PAGE and subjected to far-Western analysis (top) as described in Materials and Method. Western blot anti-actin was perform (bottom) as loading control. (B) Far-Western analyses from three different females were performed. 4E-BP levels at each time point are shown in arbitrary units as a percentage of level measured prior to fertilization. (C) Affinity-purified proteins using a m7GTP column prior (lane 1) and 60 minutes following fertilization (lane 2) were subjected to Western blot using anti-eIF4E (top panel) or anti-4E-BP2 (bottom panel) antibodies. Equal amount of eIF4E has been loaded in each lane (see Materials and Methods). (D) Equal amount of 4E-BP immunoprecipitated as described in Fig. 3A from unfertilized eggs (lane 1), 30 and 60 minutes postfertilization (lane 2 and 3) was visualized by Western blot anti 4E-BP2 (top) and tested by far-Western for eIF-4E interaction (bottom).

existence of a second antibody-crossreacting homologue of identical molecular weight. To address this possibility, the cloning of 4E-BP is currently underway.

Our study shows that 4E-BP is involved in the early events of life, as disruption of the eIF4E/4E-BP complex occurs within minutes following fertilization. It remains to be established whether 4E-BP plays a role in each cell cycle following fertilization. Sea urchins eggs represent an elegant system that will help clarify the role of 4E-BP phosphorylation in progression through the cell cycle. Indeed, during early development stage, the cleavage of sea urchin cells are highly synchronized and synchronization is achieved naturally, without drugs that could complicate the interpretation of the results due to side effects. Another crucial factor which participates in translation initiation and whose activity is dependent on the cell cycle is eIF4E (Pyronnet, 2000; Pyronnet and Sonenberg, 2001). While eIF4E phosphorylation increases rapidly following sea urchin fertilization (Waltz and Lopo, 1987), it is hypophosphorylated at mitosis in mammalian cells. Thus, studies on

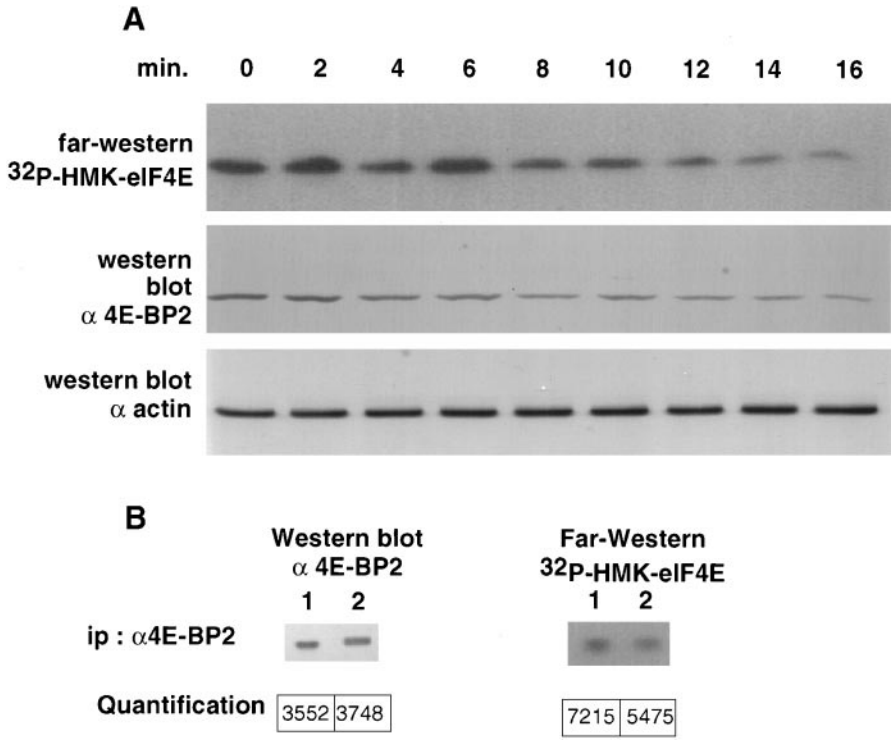

FIG. 5. The decrease in eIF4E/4E-BP association following fertilization is due to a loss of 4E-BP protein and eIF4E/4E-BP affinity. (A) 4E-BP-enriched extracts were prepared prior or every 2 minutes following fertilization (as indicated) and proteins were resolved on 15% SDS-PAGE and tested by far-Western for eIF4E binding (top) and by Western blot for 4E-BP amount (middle). A Western blot anti-actin was also performed (bottom) as loading control. (B) Equal amount of 4E-BP immunoprecipitated as described in Fig. 3A from unfertilized eggs (lane 1) and 18 minutes postfertilization (lane 2) was visualized by Western blot anti 4E-BP2 (top) and tested by far-Western for eIF-4E interaction (bottom).

sea urchin early development provide a promising model to investigate the importance of eIF4E phosphorylation through the cell cycle.

In mammals, the best candidate for eIF4E phosphorylation is Mnk1, the recently cloned MAP kinase-interacting protein kinase-1 (Fukunaga and Hunter, 1997), also called MAP kinase signal-integrating kinase 1 (Waskiewicz *et al*., 1997). In the cell, Mnk1 uses a docking site in the scaffolding protein eIF4G (Pyronnet *et al*., 1999; Waskiewicz *et al*., 1999) and phosphorylate eIF4E. Consistent with this idea,

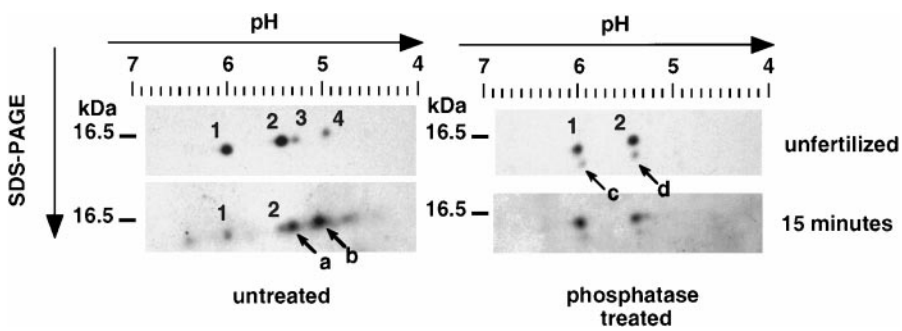

FIG. 6. 4E-BP phosphorylation is modified after fertilization. 2D gel analysis were performed with immunoprecipitated 4E-BP prior and 15 minutes following fertilization and treated or not by acid phosphatase. Proteins were resolved on IEF gel (pH 3–10) as first dimension and 12% SDS-PAGE as second dimension, followed by a Western blot using anti-4E-BP2 antibodies.

eIF4E sequestration by 4E-BP has been shown to inhibit eIF4E phosphorylation (Wang *et al*., 1998) and, conversely, eIF4E bound to eIF4G is a better substrate for phosphorylation (Tuazon *et al*., 1990; Waskiewicz *et al*., 1999; Pyronnet *et al*., 1999). Interestingly, the present study indicates that the kinetics of eIF4E/4E-BP complex disruption after fertilization follows that of eIF4E phosphorylation, almost perfectly (Waltz and Lopo, 1987). It is then reasonable that a homologue of Mnk1 bound to eIF4G exists in sea urchin and that eIF4E phosphorylation occurs following fertilization only when eIF4G brings Mnk1 in the vicinity of eIF4E. This hypothesis is further supported by the finding that PKC, which is suspected to act upstream of Mnk1 (Pyronnet *et al*., 1999), has been shown to be activated following fertilization (Olds *et al*., 1995).

4E-BP is a phosphoprotein whose hyperphosphorylation prevents its binding to eIF4E. Here, we report that at least four 4E-BP isoforms possessing distinct p*I*s exist in unfertilized eggs, and that fertilization triggers a shift from high to low p*I* isoforms. By using phosphatase treatment, we demonstrate that 4E-BP is phosphorylated to a significant extent in unfertilized eggs and that fertilization causes hyperphosphorylation. These observations are consistent with the need for a rapid phosphorylation of 4E-BP, which is responsible for eIF4E/4E-BP complex disruption detected within a few minutes following fertilization. In mammals, 4E-BP phosphorylation is an ordered, hierarchical process (Gingras *et al*., 1999b). Indeed, 4E-BP possesses primary sites whose phosphorylation is necessary for the subsequent phosphorylation of secondary sites (Gingras *et al*, 1999b). In this model, eIF4E can interact with 4E-BP phosphorylated on its primary sites, but not with the hyperphosphorylated forms. That 4E-BP is partly phosphorylated in unfertilized eggs, but still interacts with eIF4E, may suggest that primary sites similar to those of mammalian 4E-BP are conserved in sea urchin. Due to phosphorylation of these primary sites in unfertilized eggs, secondary sites could be in turn rapidly phosphorylated following fertilization and eIF4E/4E-BP complex disrupted within a few minutes. To demonstrate that such a model is correct for sea urchin, the identification of the 4E-BPs phosphorylation sites and the characterization of the signaling pathways that impinge upon their phosphorylation following fertilization are currently underway.

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